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(54) Use of cartilage-inducing factor B (clf-B) for the manufacture of a medicament for inhibiting tumor growth.

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Description

Technical Field

This invention is in the field of oncostatic compounds. More particularly it relates to using a polypeptide referred to as CIF-B or TGF-β2 for inhibiting tumour growth.

Background

European Patent Application 85304848.6 (published 22 January 1986 under publication number 0169016) describes two bovine bone-derived CIFs, designated CIF-A and CIF-B. Both have molecular weights of approximately 26,000 daltons by SDS-PAGE analysis and are dimers. They each exhibit in vitro chondrogenic activity by themselves, as measured by cartilage specific proteoglycan (PG) production in an agarose gel culture model using fetal rat mesenchymal cells. Neither, however, is chondrogenically active in vivo by itself. Amino acid sequencing of CIF-A showed that it is identical to that reported for a human placenta-derived polypeptide called beta-type transforming growth factor (TGF-β). The partial N-terminal sequence of CIF-B is different from that of TGF-β (eleven of the first 30 amino acids at the N-terminus are different). CIF-B has been determined to be a novel form of TGF-β and is sometimes called TGF-β2. Both CIFs exhibit activity in the TGF-β assay (ability to induce anchorage-independent growth of normal rat kidney cell colonies in soft agar).

EPA 86306000.0 (published 11 March 1987 under No. 0213776) discloses that both CIFs possess anti-inflammatory activity and are inhibitors of mitogen-stimulated T cell proliferation and B cell activation. It also reports that CIF is localized in centers of hematopoiesis and lymphopoiesis and that CIF may, therefore, be useful for treating indications associated with malfunction or dysfunction of hematopoiesis or lymphopoiesis.

TGF-β derived from bovine kidney, human placenta, and human platelets is described in International Patent Application PCT/US83/01460 (published 29 March 1984 under no. WO84/01106) and EPA 84450016.5 (published 19 December 1984 under no. 0128849). These applications present data showing that such TGF-β, when combined with EGF or TGF-α, promotes cell proliferation in the above mentioned soft agar culture assay and promotes cell proliferation and protein deposition in a rat soft tissue wound healing model.

TGF-β has been shown to be very similar to, if not identical to a polypeptide identified as growth inhibitor (GI) purified from BSC-1 monkey kidney cell-conditioned medium (Tucker. R. F. et al. Science (1984) 226:705). TGF-β and GI have both shown the ability to inhibit growth of a variety of tumour cell lines (Assoian, R. K. et al, Cancer Cells 3 / Growth Factors and Transformation, Cold Spring Harbor Laboratory, June 1985, pages 59-64 and Moses, H. L. et al, Cancer Cells 3 / Growth Factors and Transformation, ibid, pages 65-71).

Disclosure of the Invention

The present invention is based on the finding that CIF-B (TGF-β2) possesses oncostatic activity. Accordingly, the invention provides a new use for CIF-B, namely the use of CIF as a medicament for inhibiting tumour growth.

Brief Description of the Drawing

Figure 1 is a graph of the test results of Example 1, infra.

Modes for Carrying Out the Invention

The purification to homogeneity of CIF-B from demineralized bone and the characterization of the pure polypeptides is described in European Patent Application Publication No. 0169016.

Native bovine CIF-B is a homodimer of approximately 26,000 daltons molecular weight as measured by SDS-PAGE. Its N-terminal amino acid sequence is as follows:

```
1                5                   10
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Gln-Asp-Asn-

 15                  20                  25
-Cys-Cys-Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-

          30
-Leu-Gly-Trp-.
```

CIF-B is relatively insensitive (in terms of reduction in biological activity) to heat or trypsin treatment, but loses its activity on reduction with agents such as 2-mercaptoethanol or dithiothreitol.

The term "CIF-B" as used herein is intended to include the bovine polypeptide described above, counterpart polypeptides derived from other mammalian species such as humans, pigs, sheep, and horses and synthetic analogs (muteins) of either the bovine or other mammalian polypeptides. The analogs will typically be substantially similar in amino acid sequence (i.e., at least about 90% identify in sequence) to the particular native species). These polypeptides may be derived from native sources or be prepared by recombinant DNA technology. Recombinant polypeptide may differ from the native polypeptide in manners (e.g., lack of glycosylation) other than in amino acid sequence as is known in the art.

The oncostatic activity of CIF-B is, like the other biological activities of CIF-B, believed to be nonspecies specific. Thus, CIF-B of one mammalian species is efficacious when administered to another mammalian species. In order to lessen the likelihood of immunogenicity, however, it is preferred that the polypeptide be of the same species as the subject being treated. While the most common use of CIF-B as an oncostat will be in the treatment of humans suffering from cancer, domestic animals such as cattle, sheep and pigs, and sports or pet animals such as dogs, cats, and horses may be treated for neoplastic conditions.

CIF-B may be used as an oncostat in treating any type of cellular neoplasm, including, carcinomas, myelomas, melanomas, and lymphomas. Particularly preferred targets are breast, lung, colon and ovarian carcinomas. CIF-B may be administered locally or systemically, depending upon the nature and degree of the neoplasm being treated. For local administration an oncostatically effective amount of CIF-B formulated with a pharmaceutically acceptable carrier as an injectable for parenteral administration, or as a solid or semi-solid implant which may or may not be of a sustained or controlled release form.

Alternatively the oncostat could be delivered to solid tumours in particular, including inoperable tumours using current catheter technology for localized delivery via the arterial supply to the tumour. In this situation the oncostat could be mixed with a vasoocclusive agent, such as injectable collagen, which would provide a means to reduce perfusion of the tumour and at the same time provide for localized delivery of the oncostatic agent. Clips may also be used to occlude venous drainage, and thus maintain high doses of CIF-B in the tumour mass.

For systemic administration oncostatically effective amounts of CIF-B will be formulated with conventional carriers used for water soluble proteins (e.g., physiological saline or sugar solutions) for injection into circulation. Alternatively, they may be formulated as a sustained release formulation that releases the CIF to circulation over a prolonged time period. Specific targeting of the factor for tumour cells in systemic applications may be accomplished by conjugation of the CIF to an antibody directed against tumour specific cell surface antigen(s). Enhanced tumour cell cytotoxicity may be accomplished by covalently radiolabeling CIF-B with ^{131}I or other cytotoxic agents. CIF-B is readily iodinated and retains full biological activity. Monoclonal antibody preparations with specificity for particular tumour types, such as breast and ovarian tumours, are well known in the art. Other oncostats or chemotherapeutic drugs may be included in the formulation if desired.

The term "oncostatically effective" is intended to indicate a dose that effects a significant (> 50%) inhibition of tumour cell proliferation. In in vitro assays, 50% inhibition is generally observed at CIF-B concentrations of the order of 0.2 µg/ml and saturation is achieved at 10 µg/ml. Inhibition may be monitored in vivo by monitoring the patient's tumour burden. The amount of CIF-B which is oncostatically effective in a given treatment will depend upon the patient, the type and degree of cancer being treated and the mode of administration. In general, the amounts administered to adult humans will be in the range of 0.1 to 1000 µg.

When administered locally (e.g., to treat a solid tumour) amounts in the lower portion of this range will normally be used, typically 0.1 to 10 µg. Correspondingly systemic administration will involve the higher segment of the range (0.1-10 µg) due to clearance or other in situ inactivation of the polypeptide.

Examples

The following examples are intended to further illustrate the oncostatic activity of CIF-B. Abbreviations used in the examples are:
Gdn•HCl = guanidine hydrochloride; EDTA = ethylenediamine tetraacetic acid; CM = carboxymethyl; HPLC = high performance liquid chromatography; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM = Dulbecco's modified Eagle's medium: (^{125}I)IdUdr = 5-[^{125}I]iodo-2-deoxyuridine; DNA-deoxyribonucleic acid.

Purification of CIFs

CIF-B was purified from demineralized bone powder as in European Patent Application 85304848.6. Briefly, bovine metatarsal bone was demineralized for 16 hr in 0.5 M HCl at 4 °C and peptides solubilized utilizing a 4 M Gdn•HCl/1 mM N-ethylmaleimide/10 mM EDTA, pH 6.8 extraction procedure. CIFs were then purified by gel filtration on Sephacryl S-200 columns equilibrated in 4 M Gdn•HCl/0.02% sodium azide/10 mM EDTA, PH 6.8 followed by cationic exchange chromatography on CM cellulose using a linear 10-400 mM NaCl gradient in 6 M urea/10 mM NaCl/1 mM N-ethylmaleimide/50 mM sodium acetate, pH 4.5. Final purification and resolution of CIF-B from CIF-A was achieved by reversed phase HPLC on C_{18} columns eluted with 0-60% acetonitrile gradient in 0.1% trifluorocetic acid, pH 1.9. Homogeneity was demonstrated by silver stained SDS-PAGE analysis and by amino terminal amino acid sequence analysis.

In Vitro Assay System

Cell lines were cultured on 96-well tissue culture plates at a concentration of 3×10^3 cells per 50 µl of DMEM containing 10% fetal calf serum. Samples tested were in 0.2 M acetic acid and were lyophilized in sterile 12 × 75 mm tubes for the assay. Samples were resuspended in DMEM with 10% fetal calf serum, the appropriate dilutions made, and were added in 50 µl to the test wells in triplicate 5 hr after plating. After incubation at 37 °C in a humidified 5% CO_2-95% air atmosphere for 72 hr (^{125}I)IdUdr, a thymidine analogue was added in 10 µl of medium (37.10^{-15} Bg/ml or 10 µCi/ml). The cells were incubated an additional 24 hr and at the end of that period were washed 1X with phosphate buffered saline, fixed for 10 min in 200 µl of methanol, and air dried for 15 min. The growth of the cells was measured by the incorporation of (^{125}I)IdUdr into the DNA of the cells. The cells were solubilized in 200 µl of 1 M NaOH for 20 min at 60 °C and labelled material collected using the Titertek Supernatant Collection System. Inhibition-stimulation of growth was expressed in the percent decrease or increase of (^{125}I)IdUdr incorporation of the treated cells when compared to the incorporation of untreated cells.

Soft Agar Assay

Assays were carried out in DMEM containing 10% fetal calf serum as described by Iwata, K.K., et al, Canc. Res. (1985) 45:2689-2694. A 1 ml base layer of 0.5% agar was poured into 6 well plates. Sterile, lyophilized test samples were resuspended in 750 µl of medium containing cells (2×10^4 cells/ml) and 0.3% agar. The mixture was poured onto the base layer and allowed to harden for 15 min at room temperature. Plates were then incubated at 37 °C in a humidified 5% CO_2-95% air atmosphere for a period of 1-2 weeks. The wells were scored by counting the number of colonies formed in 8 random lower power fields.

Example 1

Pure CIF-A and CIF-B (as determined by SDS-PAGE and amino terminal sequence analysis) were tested at various concentrations on nonconfluent monolayer cultures (3×10^4 cells/well) of an established mink epithelial cell line (CCl 64) which had been shown previously to be sensitive to tumor-inhibiting polypeptides. Cultures were pulsed with (^{125}I)IdUdr ($3,7.10^{-15}$ Bg/ml or 1 µCi/ml) on day 4 and cultures harvested on day 5 and evaluated as described above (In Vitro Assay System). Identical tests on human platelet-derived TGF-β were carried out for comparison. The results of these tests on CIF-A and CIF-B are

shown in Figure 1. Values for % Inhibition represent the average of triplicate determinations. As shown in Figure 1, CIF-A and CIF-B elicit identical dose response curves with half maximal inhibition seen at 0.5 ng/ml; saturation (>90% inhibition) is achieved at about 10 ng/ml for both CIF-A and CIF-B. Human platelet-derived TGF-β generated cell dose response curves were similar to both CIFs. In addition, a striking change in cell morphology was observed in CCl 64 monolayer cultures as early as three days post treatment with CIFs. Again, this observed effect was indistinguishable in cultures treated with either CIF-A or CIF-B. In contrast to the untreated controls which exhibit a puffy cuboidal-like morphology, CCl 64 cells treated with 7.8 ng/ml of either CIF-A or CIF-B appear very flattened and display a phenotype very similar to that of normal, flattened lung epithelium.

Example 2

Using a concentration of CIF-B determined from the CCl 64 dose response curve (Figure 1) that gives a maximal inhibition, a variety of human and nonhuman tumor and "normal" cells were tested for their growth response to CIF-B in the in vitro assay system described above. CIF was added (>10 ng/ml) to plated cells (3000 cells/well) at day 1, pulsed with (^{125}I)IdUdr on day four and harvested on day 5. The results of these tests are reported in Table 1 below.

Table 1

Effects of CIF-B on the Growth of Different Cell Lines in Culture

Cell Line	[^{125}I]IdUdr Incorporation	
	% inhib.	% stim.
Human tumour		
A549 lung carcinoma	48	--
2981 lung adeno-carcinoma	46	--
A375 melanoma	52	--
A431 epidermoid carcinoma	23	--
MCF-7	60	--
A673 rhadomyo-sarcoma -	--	--
Normal		
(WI 38) human lung fibroblasts	--	119
(Sagamoto) human fibroblasts -	--	188
(SA_6) normal rat kidney	--	94
Nonhuman		
CCl 64 mink lung	93	--
SR Balb/C (Schmidt Rupin-RSV trans-formed)	--	--

As indicated in Table 1 relative to the various human tumour cell lines tested, the human lung carcinoma lines, A549 and 2981, exhibited the most sensitivity to inhibition, 48% and 46%, respectively. Likewise, a melanoma and breast carcinoma were also effective target cells. To a lesser extent, 24% inhibition was observed with an epidermoid carcinoma line (A431). Some lines exhibited minimum or no sensitivity to CIF-B; i.e., in the rhabdomyosarcoma line A673 (late passage), no detectable inhibition was seen at any concentration tested. The inhibition activity of CIF-B is not limited to cells of human origin, but also was (as shown in Figure 1) pronounced in the mink epithelial line CCl 64 (>90%) but minimally observed in murine

cells transformed by the Schmidt Rupin strain of Rous sarcoma virus (SR Balb/C). In contrast, some untransformed cell lines were stimulated rather than inhibited by CIF-B. Both human lung (WI 38) and Sagamoto fibroblasts exhibited a stimulation in DNA synthesis: 119% for WI 38, and 188% for Sagamoto fibroblasts. Normal rat kidney cultures were also stimulated at concentrations which inhibited tumour cell targets.

Example 3

The activity CIF-B as an oncostat was tested on human lung carcinoma cells (A549) using the Soft Agar Assay described above. The cells (2×10^4) were mixed with homogeneous preparations of CIF-B at various concentrations. The results are reported in Table 2 below. Values of soft agar colonies represent the average number of colonies >20 cells in diameter per eight random low power fields scored 10 days after seeding.

Table 2

Effect of CIF-B on the growth of human lung carcinoma cells in soft agar

	Soft agar colonies
Control	
(no additions)	275
CIF-B	
(ng/ml)	
0.50	237
1.0	224
5.0	114
10.0	108
20.0	54

As shown in Table 2, plates seeded with tumour cells containing as little as 1.0 ng/ml of CIF-B start to show a reduction in number of colonies. The reduction of colony size is even more striking at these low concentrations. Half-maximal inhibition in number of colonies is seen at approximately 5 ng/ml (47% reduction). At a concentration of 20 ng/ml, residual colonies observed were no more than 20-30 cells in size.

Example 4

Additional tests were carried out using the soft agar assay to evaluate the oncostatic activity of CIF-A and CIF-B on an anchorage-independent growth of primary human tumour cells in agar (Table 3). The CIFs were tested at 10 and 100 ng/ml. Four different adenocarcinomas derived from breast and ovarian tissue, one lymphoma, and one tumour of unknown etiology were tested. CIF-B proved to be the more potent oncostat in these tests, exhibiting 100% inhibition of colony formation on 3 of 4 adenocarcinomas tested and 90% inhibition on the other. The inhibition provided by CIF-A at equivalent concentrations ranged from 60-97%. CIF-B inhibited these cells 70% and 95% when tested at equivalent concentrations. The tumour cells of unknown etiology were also more effectively inhibited by CIF-B than by CIF-A.

Interestingly, several of the tumour cells tested were refractory to inhibition of proliferation by 18 different chemotherapeutic drugs, including adriamycin, platinum. and 5-fluorouracil, and yet these same cells were exquisitely sensitive to CIF-B, showing 100% inhibition at a factor concentration of 100 ng/ml.

Under conditions in which the inhibition of cell proliferation was comparable for CIF-B, and for certain chemotherapeutic drugs such as 5-fluorouracil and platinum, CIF-B was 10,000 to 100,000 more potent than the drugs on a molar basis.

Table 3

Specimen Number	Site	Type	CIF A 10	CIF A 100	CIF B 10	CIF B 100 (ng/ml)
86-0393	Breast	Adeno	79	18	30	0
86-0394		Lymphoma	31	15	5	0
86-0395	Ovary	Adeno	19	3	3	0
86-0396	Ovary	Adeno	68	39	52	10
86-0397	Ovary	Adeno	52	24	14	0
86-0399	Unkn.		51	26	31	8

Claims

1. Use of CIF-B (TGF-β2) for the manufacture of a medicament for treating cancer.

2. Use of CIF-B as in claim 1 wherein the CIF-B has the following partial N-terminal amino acid sequence:

```
1                5                   10
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Gln-Asp-Asn-

 15                  20                  25
-Cys-Cys-Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-

          30
-Leu-Gly-Trp-.
```

3. The use of CIF-B as in claim 1 or 2 wherein the cancer is a carcinoma, adenocarcinoma, melanoma, or lymphoma.

4. The use of CIF-B as in claim 3 wherein the carcinoma is breast, lung, colon or ovarian carcinoma.

Patentansprüche

1. Verwendung von CIF-B (TGF-β2) zum Erzeugen eines Medikaments zum Behandeln von Krebs.

2. Verwendung von CIF-B nach Anspruch 1, dadurch gekennzeichnet, daß das CIF-B die folgende Aminosäurenteilsequenz mit endständigem N enthält:

```
                    5                   10
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Gln-Asp-Asn-

15                  20                  25
Cys-Cys-Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-

      30
Leu-Gly-Trp.
```

3. Verwendung von CIF-B nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß der Krebs ein Karzinom, Adenokarzinom, Melanom oder Lymphom ist.

4. Verwendung von CIF-B nach Anspruch 3, dadurch gekennzeichnet, daß das Karzinom, Karzinom ein Brust-, Lungen-, Dickdarm- oder Eierstockkarzinom ist.

Revendications

1. Utilisation de CIF-B (TGF- 2) dans la préparation d'un médicament pour traiter le cancer.

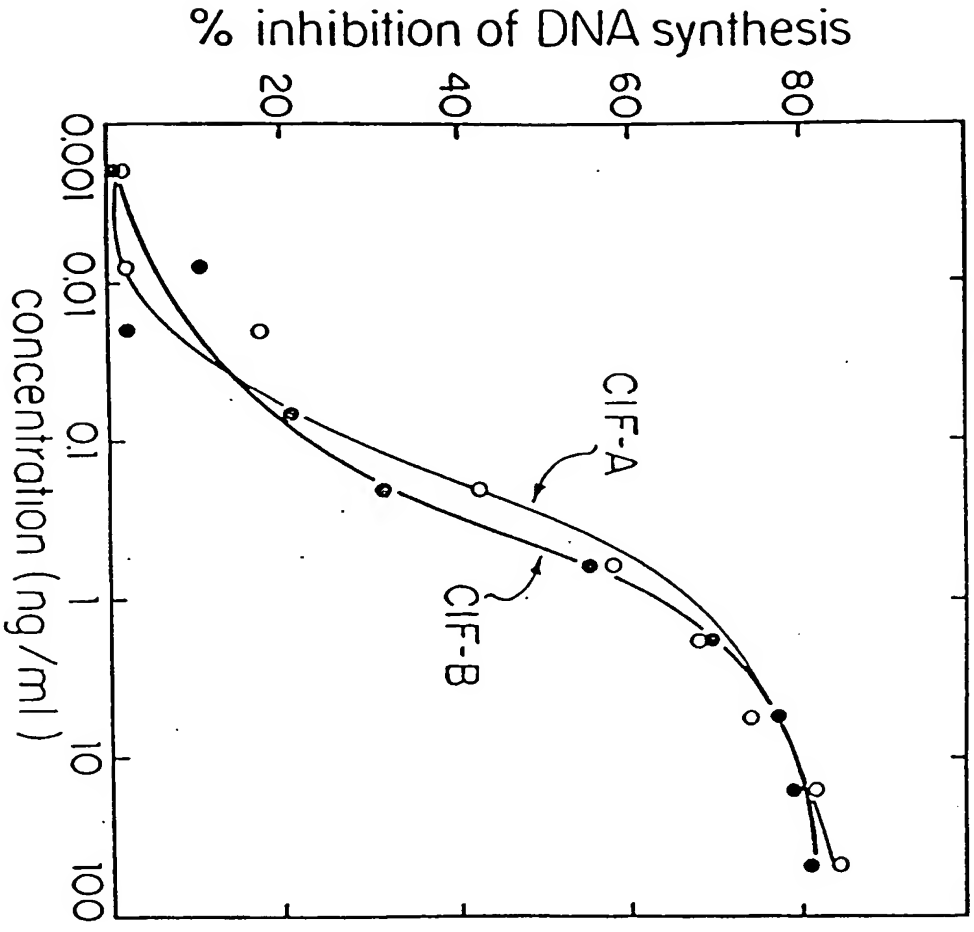
2. Utilisation de CIF-B selon la revendication 1, dans laquelle le CIF-B a la séquence partielle d'amino-acides N-terminale suivante :

```
1                   5                   10
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Gln-Asp-Asn-

 15                  20                  25
-Cys-Cys-Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-

          30
-Leu-Gly-Trp-.
```

3. L'utilisation de CIF-B selon la revendication 1 ou 2, dans laquelle le cancer est un carcinome, adénocarcinome, mélanome ou lymphome.

4. L'utilisation de CIF-B selon la revendication 3, dans laquelle le carcinome est un carcinome des seins, des poumons, du côlon ou des ovaires.

FIGURE 1


% inhibition of DNA synthesis
20
40
60
80
0.001
0.01
0.1
1
10
100
concentration (ng/ml)
CIF-A
CIF-B